APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: COMPOSITIONS FOR ISOLATING A cDNA ENCODING A MEMBRANE-BOUND PROTEIN

APPLICANT: MASAYUKI TSUCHIYA, MIKIYOSHI SAITO AND TOSHIHIKO OHTOMO

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No. EV 214 468 712 US

August 21, 2003
Date of Deposit

DESCRIPTION

NOVEL METHOD FOR GENE CLONING

Technical Field

The present invention relates to a novel gene-cloning-method for selectively and efficiently isolating genes encoding membrane-bound proteins.

Background Art

Proteins synthesized in cells can be categorized by their individual characteristics into those localized in intracellular organelles, such as nucleus, mitochondria, cytoplasm, etc.; those that function by binding to the cell membrane, such as receptors and channeling molecules; and those that function by being secreted to the cell exterior, such as growth factors and cytokines, etc. In particular, protein molecules bound to the cell membrane are responsible for biologically important functions, such as cellular responses towards growth factors and differentiation factors, inflammatory responses, cell-cell interactions, hormone responses, and so on, and therefore, can be target molecules for diagnostic and therapeutic drugs for various types of disorders.

In recent years, as typified by the genome-project, mass gene-cloning-methods employing random approaches are being conducted, and enormous gene sequence information such as large amounts of ESTs (Expressed Sequence Tags) are accumulated (Matsubara, K. Artificial Organs (1996) 20, 823-827). However, the identification of a protein having a desired function from these ESTs is by no means an easy task, and in order to predict and analyze the function of an encoded-protein from gene sequence information, a great deal of time and efforts are required. Therefore, a method to select, at least upto a certain extent, a gene encoding a protein expected to have a desired function at the stage of random cDNA cloning has been long awaited.

Cloning methods utilizing protein localization were

developed as a solution to such problems. For example, proteins secreted to the cell exterior have an amino acid sequence comprising 15 to 30 or so amino acid residues vital for secretion, which is generally termed as a secretion signal sequence or a leader sequence.

Tashiro, K. et al. focused their attention on the features of this secretory protein synthesis and developed a cloning method that specifically selects a gene encoding a secretory protein (Tashiro, K. et al., Science (1993) 261, 300-603). When the signal sequence of proteins that are normally secreted to the cell exterior, for example, interleukin-2 (IL-2) receptor, is deleted, they are unable to express on the cell membrane. If the cDNA encoding the secretion signal sequence is fused, this IL-2 receptor can be re-expressed on the cell membrane as a fusion protein. Since IL-2 receptor fusion protein-expressing cells can be selected by an antibody recognizing the IL-2 receptor, cDNA encoding the protein of which the signal sequence introduced to cells have functioned can be isolated. This method is generally called the SST (Signal Sequence Trap) method as it selectively clones a gene encoding a signal sequence. A cloning method for yeasts has also been developed by basically the same principle (U.S. Patent No. 5,536,637).

However, even if a gene fragment encoding a protein comprising a signal sequence is obtained by this method, one cannot know whether it is a secretory protein, or whether it is a membrane-bound protein. Also, this method requires the utilization of a cDNA library comprising a 5' end, but techniques for efficiently constructing a cDNA library that selectively contains a 5' end are not necessarily easy, versatile techniques.

Recently, Ishihara et al. and Nakauchi et al. reported the TMT (Transmembrane Trap) method, which more selectively clones a gene encoding a membrane-bound protein (Yoshikazu Ichihara and Yoshikazu Kurozawa, Abstracts from the Annual Meeting of the Molecular Biology Society of Japan (1998), No. 3-509-P-533, Nakauchi et al. WO98/03645). The method of Ichihara et al. is based on a principle opposite to the above-mentioned SST method.

Namely, the extracellular region of the IL-2 receptor and a protein containing the cell membrane-bound region encoded by cDNA are fused, the IL-2 receptor is expressed on cell membrane surface, and the cells are selected using an antibody against the IL-2 receptor. A model experiment of this method confirmed the expression of fusion molecules between type I or type II membrane-bound proteins, or glycosylphosphatidylinositol (GPI) anchor-type membrane-bound protein and IL-2 receptor on the cell membrane using the anti-IL-2 receptor antibody.

However, when the cDNA library was introduced, proteins not comprising the transmembrane region and membrane-bound region were also obtained within the selected cDNA. In other words, the cloning selectivity of the gene encoding the membrane-bound protein obtained by this TMT method is not necessarily high. This shows, for example, that although all fusion proteins not having the transmembrane region and GPI anchor should be secreted in principle, non-specific agglutinations not owing to the transmembrane region and GPI anchor may also occur on the cell membrane depending on the structures and amino acids compositions of the fusion proteins.

Furthermore, in the case of this TMT method, an epitope recognized by the antibody is expressed in the fusion protein. Therefore, even if fusion proteins expressed in the above manner are non-specifically adsorbed onto the cell membrane, the antibody will recognize and bind to the epitope as long as the epitope is exposed. Also, those molecules on the membrane surface that are on their way to being secreted to the cell exterior are also recognized by the antibody. Therefore, it is desired that the selectivity of membrane-bound protein-expressing cells obtained by this TMT method be further improved.

Disclosure of the Invention

The present invention solves the problems of the TMT method and provides a gene cloning method with a superior selectivity.

A feature of the present invention is to isolate a gene encoding a membrane-bound protein by linking a functional protein

to the fusion protein itself, differing from the conventional TMT method that carries an epitope recognizing an antibody. The present method thus enabled the selective isolation of genes encoding membrane-bound proteins.

Namely, the present invention provides:

(1) a method for isolating a gene encoding a membrane-bound protein, the method comprising the steps of

(i) introducing into cells a vector comprising a DNA comprising a DNA encoding a secretable, functional protein having a binding affinity to an antigen and a cDNA ligated downstream of the 3′ side of the functional protein-encoding DNA,

(ii) expressing within cells, the fusion protein of the secretable, functional protein having a binding affinity to the antigen and the protein encoded by the cDNA,

(iii) selecting cells binding to the antigen by contacting cells expressing the fusion protein on the cell membrane with an antigen, and

(iv) isolating cDNA inserted within the vector from the selected cells,

(2) the method of (1), wherein the vector introduced into cells in step (i) is obtained by introducing cDNA into a vector at the restriction enzyme site downstream of the 3′ side of the functional protein-encoding DNA,

(3) the method of (1), wherein the vector introduced into cells in step (i) is obtained by introducing into a vector, a DNA comprising a DNA encoding a functional protein and a cDNA ligated downstream of the 3′ side of the functional protein-encoding DNA,

(4) the method of any one of (1) to (3), wherein the DNA encoding the functional protein and the cDNA downstream of the 3′ side thereof are ligated via a DNA encoding a peptide linker,

(5) the method of any one of (1) to (4), wherein the cDNA is derived from a cDNA library obtained from mammalian cells,

(6) the method of any one of (1) to (5), wherein the vector introduced into cells in the step (i) comprises a DNA encoding a secretion signal sequence upstream of the 5′ side of the DNA

encoding a functional protein,

(7) the method of any one of (1) to (6), wherein the functional protein is an antibody,

(8) the method of any one of (1) to (7), wherein the functional protein having a binding affinity to the antigen is a single-chain antibody, which is preferably monovalent or bivalent,

(9) the method of any one of (1) to (8), wherein the vector contains a DNA in which a DNA encoding the constant region of the antibody is ligated downstream of the 3' side of the DNA encoding a single-chain antibody,

(10) the method of any one of (1) to (9), wherein the antigen is bound to a supporter,

(11) the method of (10), wherein the supporter is for cell-culturing,

(12) the method of any one of (1) to (11), comprising determining whether or not the gene obtained from cells comprises a novel sequence,

(13) the method of (12) comprising screening a cDNA library to obtain the full-length gene of the gene obtained from cells, the gene comprising a novel sequence,

(14) the method of (13) comprising isolating the full-length gene of the gene obtained from cells, the gene comprising a novel sequence,

(15) a kit for isolating a gene encoding a membrane-bound protein, the kit comprising a vector having a restriction enzyme recognition site for inserting a cDNA downstream of the 3' side of a DNA encoding a secretable, functional protein having a binding affinity to an antigen, and,

(16) the kit of (15) further comprising a supporter to which an antigen is bound and/or cells into which a vector is to be introduced.

As membrane-bound proteins isolatable by the method of the invention, for example, type I or type II membrane-bound proteins and GPI anchor-type membrane-bound proteins and such can be given. Type I or type II membrane-bound proteins are proteins comprising transmembrane regions, and bind to the membrane after being

secreted to the cell exterior from N terminal side or C terminal side of the expressed polypeptides. Transmembrane regions are regions that penetrate the inside and the outside of the cell membrane, and because this transmembrane region remains in the cell membrane, proteins exist as being fixed onto the cell membrane. The transmembrane region is generally constituted of hydrophobic amino acid residue-rich regions within the amino acid sequence of the protein. A commercially available computer program, for example, the GCG Sequence Analysis Software Package (Genetic Computer Group, Oxford Molecular Group, Inc.) can easily predict whether a protein has a transmembrane region or not. GPI anchor type membrane-bound proteins are proteins that undergo modifications by GPI and that are anchored to the lipid layer of the cell membrane via GPI (GPI anchor type membrane-bound proteins).

In the first step ((i)) of the isolation method of the invention, a vector comprising a DNA encoding a secretable, functional protein having a binding affinity to an antigen and a DNA wherein a cDNA is ligated downstream of the 3′ side thereof, is introduced into cells.

"A functional protein having binding affinity to an antigen" means a protein that can functionally bind to a certain antigen. As functional proteins, those of which the binding constant with the antigen is 10^7M or more are preferable. It is more preferably 10^8M or more, and is even more preferably 10^9M or more. Functional proteins are, specifically, antibodies, antibody fragments, single-chain antibodies, etc. Antibodies comprise two heavy chains (H chain) and two light chains (L chain), and these H chains and L chains bind via disulfide bonds to make a single antibody molecule. H chain and L chain are composed of a variable region (v region, Fv) and a constant region (C region, Fc). Antibody fragments are partial proteins of antibodies having a binding affinity to antigens, and, for example, Fab, F(ab′)2, Fv and such can be given. A single-chain antibody (hereafter called, single-chain Fv (scFv)), is a protein having a binding affinity to an antigen, the protein in which the H chain

Fv and L chain Fv are ligated by a linker, and, for example, a monovalent single-chain antibody and a bivalent single-chain antibody can be given. Monovalent single-chain antibodies have an antigen-binding site comprising one H chain Fv and L chain Fv, and bivalent single-chain antibodies have a structure in which two monovalent single-chain antibody molecules are ligated via a linker, and have two antigen-binding sites.

Antibodies, antibody fragments, or single-chain antibodies may be those wherein one or more amino acid residues have been deleted, inserted, and/or replaced with other amino acid residues for various purposes, such as improving the binding constant, or those which are fused with other peptides or polypeptides, and both are encompassed in the functional protein of the present invention. Also, modified antibodies may be used as the antibody, antibody fragment, or single-chain antibody. Examples of modified antibodies are chimeric antibodies and humanized antibodies. Chimeric antibodies are those comprising a V region and C region of antibodies derived from different animals. Humanized antibodies are those comprising complementarity determining region (CDR) of an antibody derived from an animal other than humans, and the framework region (FR) and the C region of an antibody derived from humans.

An antigen having binding affinity to the functional protein of the invention may be any substance as long as it has antigenicity. Examples are, proteins, peptides, and sugars and such, preferably proteins. Proteins used as antigens are, for example, cells or microorganisms expressing proteins, serum proteins, cytokines, intracellular proteins, membrane proteins, etc.

DNA encoding the antibody can be obtained by well-known means. Namely, they can be isolated from antibody-producing cells, for example, hybridoma, immortalized lymphocytes sensitized by an antigen, and cells producing a recombinant antibody following the introduction of an antibody gene. In addition, DNA that have been already isolated and inserted into a vector may also be used. The origin and type of the DNA encoding

the antibody are not questioned as long as it can be used in the present invention.

DNA encoding an antibody fragment or single-chain antibody can be constructed from DNA encoding the antibody by following methods usually employed. DNA encoding a monovalent single-chain antibody is obtained by ligating DNA encoding the H chain V region (H chain Fv) of the antibody, DNA encoding the linker, and DNA encoding the L chain V region (L chain Fv). The linker is not restricted as long as it can sterically reproduce the H chain Fv and L chain Fv so that they have an antigen affinity. Preferably it is a peptide linker and, for example, comprises 12 to 19 amino acid residues (Huston, J. S. et al., Proc. Natl. Acad. Sci. U.S.A. (1988) 85, 5879-5883). Specifically, a peptide linker having the following amino acid sequence can be given: GlyGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer ($(Gly_4Ser)_3$) (SEQ ID NO: 1). DNA encoding a bivalent single-chain antibody is constructed by linking the 5′ end and 3′ end of two DNA molecules encoding a monovalent single-chain antibody using a DNA encoding a peptide linker. The peptide linker ligating two single-chain antibodies comprises, for example, the amino acid sequence of GlyGlyGlyGlySerGlyGlyGlyGlySerGlyGlyGlyGlySer ($(Gly_4Ser)_3$) (SEQ ID NO: 1).

In order to increase the cloning efficiency in the invention, for example, when using single-chain Fv as the functional protein, it is preferable that the C terminus contains a small amount of hydrophobic amino acids, and specifically, a single-chain Fv in which the elbow region has been deleted as described in Examples below can be used. Also, it is preferable that, in the present invention, stability and expression efficiency can be increased by ligating further a domain of secretory protein origin, for example, a DNA encoding amino acids of the constant region of an antibody described in Examples below, to the C terminus of single-chain Fv.

For a functional protein to be secretable, a secretion signal sequence can be used. Namely, it is enough to ligate a DNA encoding a secretion signal sequence upstream of the 5′ side

of a DNA encoding a functional protein having a binding affinity to an antigen. As a secretion signal sequence, one that is suitable for cells used for the expression of a cDNA library and the secretion of proteins, is employed. The secretion signal sequence may be a signal sequence of any secretory protein as long as it can secrete the functional protein. Preferable animal-derived secretion signal sequences are those deriving from mammals, for example, the signal sequence of human immunoglobulin (Kabat, E. et al., Sequences of Proteins of Immunological Interest, US Department of Health and Human Services (1991)), of cytokines, and of cytokine receptors.

cDNA ligated downstream of the 3' side of a DNA encoding the functional protein preferably derives from a cDNA library. As the cDNA library, one obtained using well-known methods, or one that is commercially available may be used. A cDNA library can be prepared by isolating mRNA from desired samples and synthesizing cDNA from the isolated mRNA.

Sources from which mRNA could be isolated are, for example, mammals, animals other than mammals, plants, yeasts, bacteria, or blue-green algae, and preferably, mammals are used. Humans, monkeys, rabbits, rats, mice and such can be given as examples of mammals, and especially humans are preferable. Animals other than mammals are, for example, insects such as fruit flies (*Drosophila*), etc.

Sources from which mRNA could be isolated may be any sources, for example, cells obtained from a living body, established cell lines, embryos, tissues, blood, or organs. Representative examples are osteoblasts, hematopoietic stem cells, smooth muscle cells, neurons, stromal cells, ES cells, liver, intestine, lung, kidney, lymph nodes, etc.

Isolation of mRNA could be done by suspending the samples for isolation under the presence of a commonly used buffer by commonly used methods. To prepare whole mRNA as the first step of mRNA isolation, for example, the guanosine ultracentrifugation method (Chirgwin, J. M. et al., Biochemistry (1979) 18, 5294-5299) or the AGPC method (Chomczynski, P. and Sacchi, N., Anal.

Biochem. (1987) 162, 156-159) and such could be employed. Next, for purifying mRNA from the whole mRNA, for example, the mRNA Purification Kit (Pharmacia) and such could be used. For example, QuickPrep mRNA Purification Kit (Pharmacia) may also be used as a commercially available kit for concentrating mRNA through affinity purification using oligo dT.

cDNA is synthesized from the obtained mRNA using reverse transcriptase. Commercially available reverse transcriptase could be used. Single-stranded cDNA complementary to the mRNA could be synthesized by using an oligo dT primer complementary to the poly A of mRNA, or using an oligonucleotide of a random sequence as the primer. For example, the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation) and such may be utilized to synthesize cDNA. Double-stranded cDNA is prepared from the obtained single-stranded cDNA by DNA polymerase.

Furthermore, the cDNA library can also be selectively condensed for a specific purpose using commonly used methods. For a specific purpose, for example, for obtaining cDNA of a gene in which the expression amount varies, the differential cloning method (Lau, L.F. et al., and Nathans, D. EMBO J. (1985) 4, 3145-3151), the differential display method (Liang, P. and Pardee, A.B. Science (1992) 257, 967-971), the subtractive cloning method (Nucleic Acids Research (1988) 16, 10937), or the serial analysis gene expression method (SAGE method) (Velculescu, V.E. et al. Science (1995) 270, 484-487) may be utilized. The SST method (Tashiro, K. et al., Science (1993) 261, 300-603) and the method described in U.S. Patent No. 5,536,637 may also be utilized to condense cDNA encoding a secretory protein.

Vectors may be any vectors as long as they can transform cells and express the DNA contained therein. It is preferable to select, as an expression vector, a vector that can operate in cells to be transformed. Examples of expression vectors are plasmid vectors and virus-derived vectors.

The obtained cDNA is ligated to a vector. At this instance, cDNA can be introduced into the vector by introducing it

downstream of the 3′ side of a functional protein encoding-DNA that is already contained in the vector. For this purpose, a suitable restriction enzyme site, for example, a multi-cloning site is designed downstream of the 3′ side of the DNA encoding the functional protein, and the cDNA is introduced into that site. Also, cDNA may be ligated first downstream of the DNA encoding the functional protein, and then the obtained DNA may be introduced into the vector. The DNA construct can be introduced into a suitable restriction enzyme site comprised in a vector DNA. When preparing the vector, the DNA encoding the functional protein and the cDNA located downstream of the 3′ side may be directly ligated, or may be ligated via a DNA encoding a peptide linker to enable easy binding of the functional protein to the antigen.

The expression vector preferably contains an expression-regulating region needed for the expression of a desired DNA in cells. Promoters/enhancers can be given as expression regulating regions, and specifically, the human EF1α promoter、HCMV promoter, or SV40 promoter and such can be given. Expression vectors prepared in such a manner can be introduced into cells using commonly used methods. Examples of such methods are, the electroporation method (EMBO J. (1982) 1, 841-845), the calcium phosphate method (Virology (1973) 52, 456-467), liposome method, DEAE dextran method, etc.

A cell that is subjected to transformation could be any cell as long as the secretion signal sequence and expression regulating region contained in the vector functions within the cell, and preferable are, animal cells, for example, COS, CHO, or BAF3, etc.

In the second step ((ii)) of the method of the invention, a fusion protein of a secretable, functional protein having a binding affinity to the antigen and a protein encoded by a cDNA is expressed within cells. Specifically, cells are transformed using a vector containing DNA encoding the above-mentioned fusion protein, and are cultured under conditions suitable for cell growth. The culture is conducted according to commonly used methods. For example, DMEM, MEM, RPMI1640, and IMDM can be used as the culture medium and may be used together with serum-

supplementing solutions such as fetal calf serum (FCS).

In order to express DNA within cells, a system that induces DNA expression can be used. For example, if expression regulating systems using tetracycline, or promoters/enhancers that are expressed in response to stimulations such as, cytokines, lipopolysaccharide (LPS), steroid hormones and such are used, it is possible to induce expression of DNA within cells by stimulating the cells. When DNA is expressed, a fusion protein containing gene products of the functional protein and cDNA is produced. When the cDNA encodes a membrane-bound protein, the secretion signal sequence is eliminated at the process when the fusion protein is synthesized on the rough endoplasmic reticulum (ER) and the fusion protein is expressed on the cell membrane. When DNA encoding a peptide linker is ligated between DNA encoding a functional protein and cDNA, a fusion protein comprising the peptide linker between the functional protein and cDNA is expressed.

The third step ((iii)) of the method of the invention involves selecting a cell binding to an antigen by contacting cells expressing a fusion protein on the cell membrane with the antigen. The antigen is preferably bound to a supporter. Examples of supporters are those for cell-culture, and preferably plates, such as plastic plates, multi-well plates, culture plates, or beads. Magnetic beads can be used as beads. The antigen can be bound to the supporter using commonly used methods. For example, the antigen can be bound to the supporter by adding the antigen to a plate in the presence of a suitable buffer, leaving overnight, and washing. The antigen may be bound to the supporter via an antibody that specifically binds to the antigen. For example, after an antibody specifically binding to an antigen is added to and fixed on the plate , the antigen can be added to bind it to the supporter. Alternatively, an antigen that is not bound to the supporter and a cell may be bound first, and then, the cell can be bound to the supporter using an antibody that specifically binds to the antigen immobilized upon the supporter. After binding the antigen unbound to the supporter and the cell, the

antigen and cell can be crosslinked by crosslinking agents such as DMS (dimethylsulberimidate), BS^3 (bis(sulfosuccinimididyl) suberate, and DSS (disuccinimidyl suberate).

Cells unbound to the antigen are removed and cells bound to the antigen can be selected by incubating the plate under conditions where the cells can bind to the antigen on the plate and by washing the plate under suitable conditions after the cells are bound to the antigen. Flowcytometry (FACS) can also be used to select cells bound to the antigen. Cells selected by such methods are collected. By repeating these methods two to several times, the desired cells can be more selectively obtained.

Step four ((iv)) of the method of the invention involves isolating cDNA inserted within the vector from the selected cells. First, the vector is extracted from the cells bound to the plate, in which the vector has been introduced, and cDNA contained in the vector is isolated. When a plasmid vector is used, the plasmid vector is extracted, introduced into *E. coli*, amplified therein, and prepared to isolate cDNA. Next, the nucleotide sequence of the isolated gene is determined. Alternatively, a PCR primer is designed based on the nucleotide sequence on the vector, cDNA is amplified using this, and the nucleotide sequence is determined. When a retrovirus vector is used, cDNA is amplified by PCR in a similar manner, and the nucleotide sequence is determined.

The method of the present invention may include the step of analysis for determining whether the gene isolated above comprises a novel sequence or not. The novelty of the isolated DNA sequence may be analyzed by searching the homology of the sequence (the equivalence of the amino acid residues) using a DNA database, for example, GENBANK, EMBL, etc. The algorithm described in "Wilbur, W.J. and Lipman, D.J., Proc. Natl. Acad. Sci. USA (1983) 80, 726-730" may be followed to determine the homology of a protein.

The method of the present invention may also include the step of screening a cDNA library to obtain the full-length gene of the gene isolated above. Following commonly used methods, a cDNA library can be screened as follows. First, a fragment of the

isolated gene is labeled, used as a probe, and hybridized to the cDNA library. The cDNA clone bound to the fragment of the isolated gene is then detected using the label.

The method of the present invention can also include the step of isolating the full-length gene of the gene isolated above. This can be done by screening the cDNA library as mentioned above, isolating cDNA clones detected by methods commonly known, and determining the nucleotide sequence thereof.

Furthermore, the present invention comprises a kit used for isolating a gene encoding the above-mentioned membrane-bound protein. The kit of the invention includes a vector having a restriction enzyme recognition site for inserting a cDNA downstream of the 3' side of a DNA encoding a secretable, functional protein having a binding affinity to an antigen. The kit of the invention preferably further includes, a supporter to which an antigen is bound and/or cells into which the vector is to be introduced. Additionally, wash solutions for panning, crosslinking agents for bridging cells with the antigen, a cDNA library, solutions for collecting DNA by dissolving the selected cells and such may also be contained.

Brief Description of the Drawings

Fig. 1 schematically shows the structure of the expression cloning vector pTMT-SR345. "SR345" in the figure indicates human IL-6 receptor extracellular region, "NEO^r" the neomycin resistant gene, "EF1α" the promoter/enhancer region of peptide chain elongation factor 1α, "SV40E" the SV40 early promoter/enhancer, and "Amp^r" the ampicillin resistant gene.

Fig. 2 schematically shows the structure of the expression vector pTMT-scFv. "scFv" in the figure indicates single-chain antibody, and "Ig's" the antibody secretion signal peptide. The other symbols are the same as Fig. 1.

Fig. 3 shows the colony number recovered by panning using COS-7 cells into which various types of plasmid DNA have been introduced.

Fig. 4 schematically shows the structure of the expression

vector pTMT-BvGS3. "hPM1-BvGS3" in the figure indicates bivalent single-chain antibody. The other symbols are the same as Fig. 1 and Fig. 2.

Fig. 5 shows a histogram obtained when the COS-7 cells into which various types of plasmid DNA have been introduced were analyzed by a flow cytometer using a rabbit polyclonal antibody against humanized PM-1 antibody.

Fig. 6 shows a histogram obtained when the COS-7 cells into which various types of plasmid DNA have been introduced were analyzed by a flow cytometer using mouse anti-IL-6 receptor antibody MT-18.

Fig. 7 schematically shows the structure of the expression vector pTMT-shPM1F-K. "shPM1-Kappa" in the figure indicates a single-chain antibody. The other symbols are the same as Fig. 1 and Fig. 2.

Best Mode for Carrying out the Invention

The cloning method of the invention can be specifically carried out as described below, but the present invention is by no means restricted thereto.

Example 1: Construction of expression cloning vector pTMT-SR345

Expression cloning vector pTMT-SR345 was constructed. SR345, encoded by the DNA contained in expression cloning vector pTMT-SR345, is the extracellular region portion of human IL-6 receptor, and consists of 345 amino acid residues from the N terminus. In the expression cloning vector pTMT-SR345, the protein encoded by cDNA inserted downstream of the DNA encoding SR345 is expressed as a fusion protein with SR345. The nucleotide sequence of SR345 is shown in SEQ ID NO: 2 together with the amino acid sequence.

First, in order to amplify the app. 1.1 kb fragment containing the cDNA encoding SR345 from the cDNA of IL-6 receptor (Yamasaki, K. et al, Science (1988) 241, 825-828), PCR primers IL6R1 (SEQ ID NO: 3) and IL6R2 (SEQ ID NO: 4) were designed. A

PCR reaction mixture (100 ml) containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 100 pmol each of the above-mentioned primers, 100 ng of template DNA (cDNA encoding IL-6 receptor), and 5 units of AmpliTaq Gold enzyme was subjected to denaturation at 94°C, incubated 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and finally, incubated for 10 min at 72°C. The amplified DNA fragment was collected and purified by 1% low melting point agarose gel electrophoresis, digested by EcoRI, and inserted into the EcoRI site of expression vector pCOS1. This was transfected into *E. coli*, and plasmids were prepared to obtain those in which the DNA fragment was inserted in the right direction. The expression vector pCOS1 was constructed from plasmid HEF-PMh-g γ 1 (see WO92/19759) by deleting contained genes by EcoRI and SmaI digestion, and ligating with EcoRI-NotI-BamHI Adaptor (TaKaRa).

Next, the EcoRI site in the upstream side of SR345 was removed by the following method. First, the plasmid was partially digested by EcoRI, and a linear molecule obtained by cleavage at one site was collected. This was blunt-ended by DNA polymerase I (Klenow fragment), self-ligated, and transfected into *E. coli* to obtain expression cloning vector pTMT-SR345. The structure of the expression cloning vector pTMT-SR345 is shown in Fig. 1.

Example 2: Construction of expression vector pTMT-scFv

Expression vector pTMT-scFv was constructed. The single-chain antibody (scFv) encoded by the DNA contained in the expression vector pTMT-scFv was designed using the variable region of the humanized monoclonal antibody PM-1, which recognizes human IL-6 receptor, and a linker region. In the expression vector pTMT-scFv, the protein encoded by the cDNA inserted downstream of the DNA encoding scFv, is expressed as a fusion protein with scFv. The nucleotide sequence of scFv gene is shown in SEQ ID NO: 5 together with the amino acid sequence.

1) Amplification of the DNA fragment encoding antibody V region

The genes of humanized PM1 antibody H chain and L chain V region (Sato, K et al, Cancer Res. (1993) 53, 851-856) were

amplified by PCR. Backward primer TMT1 (SEQ ID NO: 6) for H chain V region was designed in such a manner that it should hybridize to DNA encoding the N terminus of H chain V region and comprise a SalI restriction enzyme recognition site. Forward primer LINK1 (SEQ ID NO: 7) for H chain V region was designed in such a manner that it should hybridize to DNA encoding the C terminus of H chain V region and comprise 5′ end sequence of a linker region. Also, backward primer LINK3 (SEQ ID NO: 8) for L chain V region was designed in such a manner that it should hybridize to DNA encoding the N terminus of L chain V region and comprise 3′ end sequence of a linker region. Forward primer SCP-C (SEQ ID NO: 9) for L chain V region was designed in such a manner that it should hybridize to the nucleotide sequence encoding the amino acid sequence forming L chain constant region elbow site, and also comprise HindIII restriction enzyme recognition site, nucleotide sequence encoding FLAG peptide (SEQ ID NO: 10), and two repetitive translation stop codons.

A PCR reaction mixture (100 ml) containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, 100 pmol each of the above-mentioned primers, 100 ng of template DNA, and 5 units of AmpliTaq Gold enzyme was subjected to denaturation at 94℃ for 9 min, incubated 30 cycles of 30 sec at 94℃ and 1 min at 60℃, and finally, incubated for 5 min at 60℃. The PCR product was purified using a 1.5% low melting point agarose gel.

2) Amplification of the DNA fragment encoding a linker region

The DNA fragment encoding a linker region comprising the amino acid sequence of $(Gly_4Ser)_3$ was amplified by the PCR method using humanized single-chain antibody expression vector pSCFVT7-hM21 (see WO95/14041). Backward primer LINK2 (SEQ ID NO: 11) was designed in such a manner that it should hybridize to the 5′ end of the linker region, and also comprise the 3′ end DNA sequence of H chain V region. Forward primer LINK4 (SEQ ID NO: 12) was designed in such a manner that it should hybridize to 3′ end of the linker region, and also comprise the DNA sequence of 5′ end of L chain V region. PCR was conducted using 100 ng of

template DNA(pSCFVT7-hM21) under the conditions above-mentioned, and the PCR product was purified using a 1.5% low melting point agarose gel.

3) Construction of humanized PM1 antibody single-chain Fv

The DNA fragment encoding H chain and L chain V regions prepared above, and the DNA fragment encoding the linker region were assembled by the PCR method, and backward primer TMT1 and forward primer TMT2 (SEQ ID NO: 13) were added to amplify the full-length DNA fragment encoding scFv of humanized PM1. The forward primer TMT2 was designed in such a manner that it should hybridize to the DNA sequence encoding HindIII restriction enzyme recognition site and FLAG peptide, and also comprise two repetitive translation stop codons, and the EcoRI restriction enzyme recognition site. The primary PCR was conducted as follows: 98 ml of a PCR reaction mixture containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 0.1 mM dNTPs, 1.5 mM $MgCl_2$, app.100 ng each of the above PCR products, and 5 units of AmpliTaq Gold enzyme was subjected to denaturation first at 94℃, and then 2 cycles of 2 min at 94℃, 2 min at 55℃, and 2 min at 72℃ were done to ligate each DNA fragment. The secondary PCR was done in the following manner: 100 pmol of each primer was added to the above PCR reaction solution, 30 cycles of 30 sec at 94℃ and 1 min at 60℃ were done, and finally, the mixture was incubated for 5 min at 60℃.

After the PCR product was purified using a 1.5% low melting point agarose gel, it was digested by SalI and NotI, and inserted into expression vector pSFLAG comprising human EF1α promoter and the leader sequence of the antibody (SEQ ID NO:14). After DNA sequencing, plasmid pTMT-scFv, containing a DNA fragment comprising the correct DNA sequence, was obtained. The structure of the expression vector pTMT-scFv is schematically shown in Fig. 2. The construction of pSFLAG was done as mentioned below. Two overlapping oligonucleotides S-FLAG1 (SEQ ID NO: 15) and S-FLAG2 (SEQ ID NO: 16) in the sense and antisense directions, respectively, were designed and synthesized so as to encode EcoRI

restriction enzyme recognition site, the leader sequence of the antibody (SEQ ID NO: 14), FLAG peptide (SEQ ID NO: 10), and KpnI, NotI, and BamHI restriction enzyme recognition sites. A reaction mixture containing 100 pmol each of the synthesized oligonucleotides was incubated for 5 min at 96℃, and the temperature was lowered to 65℃ over 20 min, and incubated for 5 min at 65℃. Then, the temperature was lowered to 42℃ over 20 min, the mixture was incubated for a further 5 min, and the two oligonucleotides were annealed by lowering the temperature to room temperature over 20 min. This DNA fragment was inserted into pCOS1 digested by EcoRI and BamHI.

Example 3: Construction of SR345-gp130 and scFV-gp130 fusion protein expression systems

(A) SR345-gp130

The cytokine signal transduction molecule gp130 is a type I membrane-bound protein (Taga, T. et al., Cell (1989) 58, 573-581, Saito, M., et al., J. Immunol. (1992) 148, 4066-4071). A portion of mouse gp130 cDNA was ligated downstream of a cDNA encoding soluble-type IL-6 receptor (SR345) of the expression vector pTMT-SR345, to express a fusion protein comprising SR345 and a partial sequence of mouse gp130, in COS cells. Two types of fusion proteins were constructed according to their differences in the gp130 partial regions. One of them is a membrane-bound fusion protein (SR345-mgpTMIC) in which the transmembrane region of gp130 and the subsequent intracellular region are ligated, and the other is a secretory fusion protein (SR345-mgpIC) in which only the intracellular region of gp130 is ligated. SEQ ID NO: 17 shows the amino acid sequence and the nucleotide sequence of full-length mouse gp130.

1) Creation of the membrane-bound fusion protein SR345-mgpTMIC expression vector

The full-length mouse gp130 cDNA was digested with EcoRI to obtain an EcoRI fragment of app. 1.1 kb. This EcoRI fragment encodes amino acids from the position 603rd to the position 917th

(C terminus) of mouse gp130, and contains a portion (15 amino acids) of the extracellular region of mouse gp130 and the whole of the subsequent transmembrane region and the intracellular region. This EcoRI fragment was inserted into the EcoRI site of pTMT-SR345 expression vector to create the membrane-bound fusion protein SR345-mgpTMIC expression vector.

2) Creation of secretory fusion protein SR345-mgpIC expression vector

To obtain a cDNA fragment encoding the intracellular region of mouse gp130, the PCR primers mgp2 (SEQ ID NO: 20; including DNA encoding the 646th amino acid to the 651st amino acid of the amino acid sequence of SEQ ID NO: 17), to which the HindIII and EcoRI sites had been added, and mgp3 (SEQ ID NO: 19; including DNA encoding the 912th amino acid to the 917th amino acid (C terminus) of the amino acid sequence of SEQ ID NO: 17) were synthesized, and using these primers, an app. 1 kb cDNA fragment of mouse gp130 was obtained. This app. 1 kb cDNA fragment encodes the 646th amino acid to the 917th amino acid in the amino acid sequence of SEQ ID NO: 17, and this corresponds to the intracellular region in which six amino acids from the N terminus are lacking. The cDNA fragment thus obtained, was digested with EcoRI, and inserted into EcoRI site of pTMT-SR345 expression vector to prepare the secretory fusion protein SR345-mgpIC expression vector.

(B) scFv-gp130

In the expression vector pTMT-scFv, a portion of mouse gp130 cDNA was ligated downstream of scFv cDNA to express a fusion protein comprising scFv and a partial region of mouse gp130 in COS cells. Two types of fusion proteins were constructed according to their differences in the ligated gp130 partial regions. One of them is a membrane-bound fusion protein (scFv-mgpTMIC) in which the transmembrane region of gp130 and the subsequent intracellular region are ligated, and the other is secretory fusion protein (scFv-mgpIC) in which only the

intracellular region of gp130 is ligated.

1) Creation of membrane-bound fusion protein scFv-mgpTMIC expression vector

To obtain a cDNA fragment encoding the whole intracellular region and transmembrane region of mouse gp130, the PCR primers mgp1 (SEQ ID NO: 18; including DNA encoding the 603rd amino acid to the 608th amino acid in the amino acid sequence of SEQ ID NO: 17), to which the HindIII site had been added, and mgp3 (SEQ ID NO: 19; including DNA encoding the 912th amino acid to the 917th amino acid (C terminus) of the amino acid sequence of SEQ ID NO: 17), to which the NotI site had been added, and using these primers, an app. 1.1 kb cDNA fragment of mouse gp130 was obtained. This app. 1.1 kb cDNA fragment encodes the 603rd amino acid to the 917th amino acid (C terminus) in the amino acid sequence of SEQ ID NO: 17, and this comprises a part of the extracellular region of mouse gp130 (15 amino acids) and the whole of the subsequent transmembrane region and the intracellular region. The cDNA fragment thus obtained was digested with HindIII and NotI, and inserted into the HindIII-NotI sites of pTMT-scFv expression vector to prepare the membrane-bound fusion protein scFv-mgpTMIC expression vector.

2) Creation of the secretory fusion protein scFv-mgpIC expression vector

To obtain a cDNA fragment encoding the intracellular region of mouse gp130, the PCR primers mgp2 (SEQ ID NO: 20; including DNA encoding the 646th amino acid to the 651st amino acid in the amino acid sequence of SEQ ID NO: 17), to which the HindIII-EcoRI sites had been added, and mgp3 (SEQ ID NO: 19; including DNA encoding the 912th amino acid to the 917th amino acid (C terminus) of the amino acid sequence of SEQ ID NO: 17), to which the NotI site had been added, were synthesized, and, by PCR using these primers, an app. 1 kb cDNA fragment of mouse gp130 was obtained.

This app. 1 kb cDNA fragment encodes the 646th amino acid to the 917th amino acid (C terminus) in the amino acid sequence of SEQ ID NO: 17, and this corresponds to the intracellular region

in which six amino acids from the N terminus are lacking. The cDNA fragment thus obtained was digested with HindIII and NotI, and inserted into HindIII-NotI sites of the expression vector pTMT-scFv to prepare the secretory fusion protein scFv-mgpIC expression vector.

Example 4: Expression by COS cells

Each type of the above-mentioned expression vectors was transfected into COS cells, the fusion protein was transiently expressed, and it was verified that cells expressing the fusion protein on the cell membrane were selectively condensed by panning. COS cells transfected with the expression vector not containing genes were used as the negative control. The positive control were COS cells that were transfected with the expression vector P3.19, which was prepared by introducing DNA encoding the HM1.24 antigen protein (WO 98/14580) into the vector pCOS1, and that were panned with the corresponding antibody.

1) Transfection into COS cells

The plasmid DNA was transfected into COS-7 cells using Lipofect AMINE PLUS™ Reagent (GIBCO-BRL). Namely, COS-7 cells seeded in $1x10^5$ cells/well (6-well plate) on the day prior to the transfection were cultured overnight, and washed with serum-free DMEM culture medium (GIBCO-BRL), and then 0.8 ml of the same culture medium was added thereto. Separately, after 1 µg of plasmid DNA and 6 µl of PLUS Reagent were added to 0.1 ml serum-free DMEM culture medium, the mixture was incubated for 15 min at room temperature, mixed with 0.1 ml of LipofectAMINE solution (4 µl of LipofectAMINE/0.1 ml of serum-free DMEM culture medium) and incubated for further 15 min at room temperature. Next, this mixture was added to the above-mentioned COS-7 cells and incubated for 3 hours at 37℃. DMEM culture medium (1 ml) containing 20% fetal calf serum (GIBCO-BRL) was added thereto (final concentration 10% serum). Following an overnight culture, the culture medium was changed to 3 ml of DMEM culture medium containing 10% fetal calf serum, and incubated for 3 days under

the conditions of 37℃ and 5% CO_2.

2) Preparation of the panning-dish

When using the expression vector pTMT-SR345, a dish coated with the mouse anti-human IL-6 receptor antibody MT18 (see unexamined published Japanese patent application No. Hei 2-288898) was prepared according to the method of Seed, B. et al., Proc. Natl. Acad. Sci. USA. (1987) 84, 3365-3369. Namely, mouse anti-IL-6 receptor antibody was added to 50 mM Tris-HCl (pH9.5) to 10 μg/ml. The antibody solution thus prepared (3 ml) was incubated at room temperature for two hours in a 60 mm-diameter cell culture dish. After washing the culture dish three times with a 0.15M NaCl solution, PBS containing 5% fetal calf serum, 1 mM EDTA, and 0.02% NaN_3 was added, and then after blocking, panning was done as mentioned below.

When using pTMT-scFv, two types of panning dishes were prepared. One was coated with soluble-type IL-6 receptor (SR344) (Yasukawa, K. et al., J. Biochem. (1990) 108, 673-676), and the other was coated with the above-mentioned mouse anti-IL-6 receptor antibody. The concentration of SR344 was adjusted to 2 μg/ml with 50 mM Tris-HCl (pH9.5), and a panning dish was prepared as mentioned above. When the negative control pCOS-1 was used, a dish coated with the above-mentioned mouse anti-IL-6 receptor antibody was utilized. When the positive control HM1.24 antigen protein expression vector P3.19 was used, a dish coated with the antibody against HM1.24 antigen was utilized.

3) Panning

pCOS-1- or, pTMT-SR345-transfected COS-7 cells were detached from the plate by 1 mM EDTA containing PBS, washed once with PBS containing 5% fetal calf serum, suspended in 2 ml of PBS containing 5% fetal calf serum and 0.02% NaN_3, and added to the panning plate coated with mouse anti-IL-6 receptor antibody.

pTMT-scFv-transfected COS-7 cells were panned by three different methods. In one method, after detached as mentioned above and washed once with PBS containing 5% fetal calf serum,

COS-7 cells were suspended in 500 µl of PBS containing 2µg/ml SR344, 5% fetal calf serum, and 0.02% NaN_3, and incubated on ice for 1 hr. After washing three times with ice-cold PBS, the cells were resuspended in PBS containing 0.2 mM crosslinker bis(sulfosuccinimidyl) suberate (BS^3; PIERCE) and 50 mM Hepes (pH8.0), and further incubated on ice for 30 min. Then, 1 M Tris-HCl (pH8.0) was added to 50 mM, and incubated further on ice for 10 min to remove the excess amount of the crosslinker. After washing cells with PBS, they were added to a panning plate coated with mouse anti-IL-6 receptor antibody. In the second method, COS-7 cells preincubated with SR344 were added to the panning plate coated with mouse anti-IL-6 receptor antibody without crosslinker treatment. In the third method, COS-7 cells were added to a plate directly coated with SR344. The time of incubation on ice, Tris-HCl treatment, and washing were similar in all the three methods.

COS-7 cells transfected with the HM1.24 antigen protein expression vector P3.19 were added to a panning plate coated with the antibody against HM1.24 antigen (WO 98/14580). After incubating the above-mentioned various types of COS-7 cells on the various panning plates for 2 hr at room temperature, the plates were gently washed three times with PBS containing 5% fetal calf serum and 0.02% NaN_3, and plasmid DNAs were collected from cells bound to the panning dishes using 0.6% SDS and 10 mM EDTA-containing solution. The condensation effect due to panning was evaluated by transfection of a 1/5th of the recovered plasmid DNA into *E. coli* DH5a using electroporation, and by the number of ampicillin-resistant colonies that had appeared. The results are shown in Fig. 3.

When the expression vector pTMT-SR345 was used, SR345-mgpIC resulted in more colonies than SR345-mgpTMIC, and therefore, no specificity against the membrane-bound protein was seen. On the other hand, when using expression vector pTMT-scFv, in all panning methods, scFv-mgpTMIC resulted in more colonies than scFv-mgpIC, and therefore, cells expressing membrane-bound protein were specifically condensed. The selectivity was more evident

especially when a crosslinking agent was used.

Thus, the above-mentioned results show that cDNA encoding a membrane-bound protein was more selectively and efficiently obtained by expressing the functional protein (single-chain antibody) as a fusion protein on the cell surface than by expressing just an epitope recognized by the antibody as the fusion protein.

Generally, several repetitive pannings enhance the clone-selectivity, but, as shown by the present example, in the present invention, in which a functional protein is expressed on the cell membrane, an outstanding selectivity was observed in the first panning. Therefore, cloning of a gene encoding a membrane-bound protein can be extremely efficiently and selectively accomplished by panning further several times.

Example 5: Construction of the fusion protein expression system using humanized bivalent single-chain Fv

1. Construction of the humanized PM1 antibody bivalent single-chain Fv expression vector

A bivalent single-chain Fv expression vector was constructed based on humanized PM1 antibody Fv. The humanized PM1 antibody single-chain Fv (hPM1-BvGS3) having a bivalent variable region was designed so that two molecules of the humanized PM1 antibody single-chain Fv2 described in Example 2 were ligated via a peptide linker comprising $(Gly_4Ser)_3$ (SEQ ID NO: 1). The amino acid sequence and nucleotide sequence of hPM1-BvGS3 are shown in SED ID NO: 21.

The construction of expression vector pTMT-BvGS3 was done as follows. A gene encoding a humanized PMI antibody single-chain Fv having, in its C terminus, a linker comprising $(Gly_4Ser)_3$ was amplified by the PCR method. TMT-1 (SEQ ID NO: 6) was used as the backward primer. Also, the forward primer BvGS3 (SEQ ID NO: 22) was designed in such a manner that it should hybridize to DNA encoding the C terminus of L chain V region and also comprise the nucleotide sequence encoding the linker and restriction enzyme SalI recognition site. PCR was conducted using 100 ng of

pTMT-scFv as the template DNA under the same conditions as mentioned above, and the PCR product was purified using 1.5% low melting point agarose gel.

The purified PCR product was digested with restriction enzyme Sall, and inserted into the cloning vector pBluescriptII (Stratagene). After DNA sequencing, the plasmid containing the DNA fragment comprising the correct DNA sequence was digested with restriction enzyme SalI, to obtain a gene encoding humanized PM1 antibody single-chain Fv having, in its C terminus, a linker comprising $(Gly_4Ser)_3$. Next, by inserting the DNA fragment obtained as mentioned above into pTMT-scFv, hPM1-BvGS3 expression vector TMT-BvGS3 was obtained. The structure of the hPM1-BvGS3 expression vector pTMT-BvGS3 is schematically shown in Fig. 4.

2. Construction of fusion protein hPM1-BvGS3-gp130 expression vector

In the expression vector pTMT-BvGS3, a portion of the mouse gp130 cDNA was ligated downstream of the cDNA encoding hPM1-BvGS3 to construct a fusion protein expression system comprising hPM1-BvGS3 and a partial region of mouse gp130. Two types of fusion proteins were constructed according to their differences in the gp130 partial regions that are ligated. One of them is a membrane-bound fusion protein (BvGS3-mgpTMIC) in which the transmembrane region of gp130 and the subsequent intracellular region are ligated, and the other is secretory fusion protein (BvGS3-mgpIC) in which only the intracellular region of gp130 is ligated. BvGS3-mgpTMIC and BvGS3-mgpIC were those prepared in Example 3 (B)-1) and 2), respectively, and by inserting these into the HindIII-NotI sites of pTMT-BvGS3, membrane-bound fusion protein expression vector pTMT-BvGS3-mgpTMIC and secretory fusion protein expressing vector pTMT-BvGS3-mgpIC were constructed.

Example 6: Analysis of expression by a flow cytometer

Each type of the expression vectors constructed above, pTMT-BvGS3, pTMT-BvGS3-mgpIC, and pTMT-BvGS3-mgpTMIC, was

transfected into COS-7 cells, the fusion protein was transiently expressed, and the expression on the cell membrane was analyzed with a flow cytometer (FACScan, Beckton Dickinson). The expression analysis was conducted by two types of methods. One involved detection by a rabbit polyclonal antibody against humanized PM-1 antibody, and the other involved detection by mouse anti-IL-6 receptor antibody MT-18 in the presence of soluble IL-6 receptor antibody. As a result, it was confirmed that the membrane-bound fusion protein BvGS3-mgpTMIC was strongly expressed on the cell membrane in a form that could recognize soluble IL-6 receptor. Cells transfected with expression vector pCOS-1 only were used as the negative control.

1) Transfection into COS-7 cells

The plasmid DNA was transfected into COS-7 cells using the transfection kit FuGENE™6 (Boehringer-Mannheim).

Namely, COS-7 cells seeded in 5×10^4 cells/well (6-well plate) on the day prior to the transfection were cultured overnight under the conditions of 37℃ and 5% CO_2 in 2 ml of DMEM culture medium (GIBCO-BRL) containing 10% fetal calf serum. On the day of transfection, 6 µl of FuGENE™6 was added to 0.1 ml of serum-free DMEM culture medium and, after incubating for 5 min at room temperature, was mixed with 2 µg of plasmid DNA, and incubated for further 15 min at room temperature. Next, this mixture was added to the above-mentioned COS-7 cells and incubated for three days under the conditions of 37℃ and 5% CO_2.

2) Staining of COS-7 cells

The above-mentioned COS-7 cells were detached with PBS containing 1 mM EDTA, washed with PBS containing 5% fetal calf serum, suspended in 50 µl of FACS buffer (PBS containing 2% fetal calf serum and 0.05% NaN_3), and stained by the following two types of methods.

A) Staining with a rabbit polyclonal antibody against humanized PM-1 antibody

A rabbit polyclonal antibody against humanized PM-1

antibody (2 μg/reaction) was added to the above-mentioned COS-7 cells, incubated for 30 min on ice, washed twice with 1 ml FACS buffer, and resuspended in 50 μl of FACS buffer. Next, 2 μl/reaction of FITC (fluorescein isothiocyanate)-labeled goat anti-rabbit IgG (AMERICAN QUAREX) and, for separately staining dead cells, 2.5 μg/reaction of PI (propidium iodide) were added and incubated for 30 min on ice in the dark. After the incubation, the cells were washed twice with 1 ml of FACS buffer, and resuspended in 0.5 ml of FACS buffer to analyze with a flow cytometer.

B) Staining with mouse anti-IL-6 receptor antibody MT-18

Soluble-type IL-6 receptor (3 μg/reaction) was added to the above-mentioned COS-7 cells and incubated for 4 hr on ice, washed twice with 1 ml FACS buffer, and resuspended in 50 μl of FACS buffer. Next, 2 μg/reaction of mouse anti-IL-6 receptor antibody MT-18 was added and incubated for 30 min on ice. After the incubation, the cells were washed twice with 1 ml of FACS buffer and resuspended in 50 μl of FACS buffer. Next, 2 μl/reaction of FITC-labeled goat anti-mouse IgG2b (Dainippon Seiyaku) and, for separately staining dead cells, 2.5 μg/reaction PI (propidium iodide) were added and incubated for 30 min on ice in the dark. After the incubation, the cells were washed twice with 1 ml of FACS buffer, and resuspended in 0.5 ml of FACS buffer to analyze with a flow cytometer.

3) Analysis of expression with a flow cytometer

Analysis by PI and FSC (Forward Scatter) revealed that a population of cells stained with PI (dead cells) was present. Dead cells disturb the analysis as they are non-specifically stained with FITC. Hence, the cell-population that was not stained with PI (living-cells) was gated, and analysis was carried out for this population only.

As a result of staining with the rabbit polyclonal antibody against humanized PM-1 antibody, no expression of secretory protein hPM1-BvGS3 was seen on the cell membrane, however, the

expression of membrane-bound fusion protein BvGS3-mgpTMIC showed the strongest expression. From this fact, it is presumed that BvGS3-mgpTMIC, which has a transmembrane region, is not secreted and is trapped in the cell membrane. However, on the other hand, the expression of BvGS3-mgpIC was detected on the cell membrane, despite the fact that it is a secretory fusion protein. This is presumed to be due to the fact that the characteristics of BvGS3-mgpIC are different from that of BvGS3 in the molecular size and structure, hydrophobic region contained in the mgpIC connected downstream thereof, etc. Namely, it is presumed that, due to its difference in characteristics, BvGS3-mgpIC does not pass through the cell membrane as swiftly as BvGS3 does, and it takes more time to pass through. As a result, the amount localized on the cell membrane increases, and all whose epitopes are extruding extracellulary are detected by a rabbit polyclonal antibody against humanized PM-1. The results are shown in Fig. 5.

On the other hand, in the case of staining with mouse anti IL-6 receptor antibody MT-18, similar to the results of staining by rabbit polyclonal antibody against humanized PM-1 antibody, though expression was not detected for secretory protein hPM1-BvGS3, the expression of membrane-bound fusion protein BvGS3-mgpTMIC was the strongest. However, for the secretory fusion protein BvGS3-mgpIC, the results were different from those obtained by rabbit polyclonal antibody against humanized PM-1 antibody, and any expression was hardly detected on the cell membrane. This indicates that, while the membrane-bound fusion protein BvGS3-mgpTMIC is expressed on the cell membrane forming a functional conformation that could recognize soluble-type IL-6 receptor, most of the secretory fusion protein BvGS3-mgpIC, though localized on the cell membrane, does not have a functional conformation that could recognize soluble-type IL-6 receptor. The results are shown in Fig. 6.

Thus, the results obtained by the flow cytometer suggests that when a mere epitope recognized by an antibody is expressed as a fusion protein, even secretory fusion proteins will be

selected as false-positive if they are localized on the cell membrane. On the other hand, when the functional protein of the invention (for example, single-chain antibody) is expressed on cell surface as a fusion protein, the possibility of cloning cDNA encoding a membrane-bound protein more selectively and efficiently has been revealed.

Example 7

1. Designing of humanized PM1 antibody single-chain Fv

In order to improve the cloning efficiency, three other types of single-chain Fv and their bivalent single-chain Fv were designed. Since the elbow region (SEQ ID NO: 5, amino acid sequence from the 242nd to 256th positions), added at the time of constructing above-mentioned humanized PM1 antibody single-chain Fv, contained amino acids residues with high hydrophobicity, the following three types of humanized PM1 antibody single-chain Fv were designed for more stable extracellular expression. Namely, in order to remove the hydrophobic region in the C terminus, single-chain Fv depleted of the elbow region was designed and designated as shPM1(ΔEL) (SEQ ID NO: 23). Also, since it was perceived that the stability and expression efficiency would increase by adding a certain secretory protein-derived domain to the C terminus of single-chain Fv, the amino acid sequence encoded by human κ chain constant region or human membrane-type μ chain constant region exon 4 (Dorai, H and Gillies S.D. Nucleic Acid Res., 17, 6412, 1989) was added to the C terminus of single-chain Fv (SEQ ID NO: 23). Although the 107th amino acid residue of human κ chain constant region is originally cysteine, one replaced with serine residue (SEQ ID NO: 24) was used this time. Also, the sequence from which transmembrane region and intracellular region had been deleted (SEQ ID NO: 25) was used as the amino acid sequence encoded by human membrane-type μ chain constant region exon 4. Those in which respective sequences mentioned above were added to the C terminus of single-chain Fv were termed shPM1-Kappa (SEQ ID NO: 26) and shPM1-MCH4 (SEQ ID NO: 27).

2. Construction of the shPM1(ΔEL) expression vector

The gene encoding shPM1(ΔEL) was amplified by PCR method. The backward primer EF-1 (SEQ ID NO: 28) and the forward primer SCP-C2 (SEQ ID NO: 29) were used. PCR was conducted using 100 ng of pTMT-scFv as template DNA under the conditions mentioned above, and the PCR product was purified using 1.5% low melting point agarose gel. The forward primer SCP-C2 was hybridized to DNA encoding the C-terminus of L chain V region, and, nucleotides encoding restriction enzyme HindIII-NotI recognition sites and FLAG peptide (SEQ ID NO: 10) were added thereto.

After the purified PCR product was digested with EcoRI and NotI, the digested product was inserted into pSFLAG vector to obtain the shPM1(ΔEL) expression vector pTMT-shPM1F. Moreover, pTMT-shPM1F-BvGS3, the expression vector for shPM1(ΔEL)-BvGS3 (SEQ ID NO: 30), single-chain Fv which had a bivalent variable region and whose elbow region had been deleted, was obtained by a similar method to that in Example 5.

3. Construction of the shPM1-Kappa expression vector

The gene encoding the fusion protein of humanized PM1 antibody single-chain Fv (SEQ ID NO: 23) and human κ chain constant region (SEQ ID NO: 24) was constructed by PCR assembling. Namely, after the genes encoding humanized PM1 antibody single-chain Fv and human κ chain constant region were separately amplified by PCR, they were assembled by their complementarity and the full-length gene was amplified by the external primer.

First, the gene encoding human κ chain constant region was amplified by PCR. The backward primer Kappa1 (SEQ ID NO: 31) was designed in such a manner that it should hybridize to the nucleotide sequence encoding the elbow region of human κ chain constant region and the 12th position Pro to the 21st position Gly of the following amino acid sequence (SEQ ID NO: 24). The forward primer Kappa2 (SEQ ID NO: 32) was designed in such a manner that it should hybridize to the nucleotide sequence encoding the 101st position Ser to the 111th position Ser of human κ chain constant region C terminus (SEQ ID NO: 24) and comprise the nucleotide

sequence encoding restriction enzyme HindIII and NotI recognition sites and FLAG peptide (SEQ ID NO: 10), and two stop codons. By using these primers, 107^{th} amino acid residue of SEQ ID NO: 24, which is originally a cysteine residue, was replaced with a serine residue. PCR was conducted under the similar conditions to those of the above-mentioned manner using the two kinds of primers above-mentioned, and humanized PM1 antibody L chain expression vector RV1-PM1a (see WO92/19759) as the template DNA. The PCR product was purified using 1.5% low melting point agarose gel.

Next, the gene encoding humanized PM1 antibody single-chain Fv was amplified in the same manner. PCR was conducted under the same conditions as above-mentioned using EF1 (SEQ ID NO: 28) as the backward primer, SCP-K (SEQ ID NO: 33) as the forward primer, and pTMT-scFv as the template DNA. The forward primer SCP-K was designed in such a manner that it should hybridize to the nucleotide sequence encoding single-chain Fv C terminus shown in SEQ ID NO: 5, and comprise a nucleotide sequence that was complementary to the 5′ end of the κ chain gene amplified by PCR. The PCR product was purified in the same manner.

Using the method shown in Example 2-3), the full-length cDNA fragment encoding shPM1-Kappa was amplified. Namely, 100 ng each of the above-mentioned DNA fragments was assembled by primary PCR, and then 100 ng each of the backward primer EF-1 (SEQ ID NO: 28) and forward primer Kappa2 (SEQ ID NO: 32) was added to amplify the full-length cDNA fragment.

After purifying the PCR product with 1.5% low melting point agarose gel, it was digested with restriction enzymes EcoRI and NotI, and inserted into pSFLAG vector to obtain shPM1-Kappa expression vector pTMT-shPM1F-K (Fig. 7). Moreover, using the similar methods to those described in Example 5, a single-chain Fv having a bivalent variable region and shPM1-Kappa-BvGS3 (SEQ ID NO: 34) expression vector pTMT-shPM1FK-BvGS3 were obtained.

4. Construction of the shPM1-MCH4 expression vector

The gene encoding the fusion protein of humanized PM1 antibody single-chain Fv (SEQ ID NO: 23) and human μ chain constant

region partial sequence (SEQ ID NO: 25) was constructed by PCR assembling. Namely, the genes encoding humanized PM1 antibody single-chain Fv and human μ chain constant region partial sequence were amplified separately by PCR method, and were assembled by their complementarity. The full-length gene was then amplified by the external primer.

First, the gene encoding human μ chain constant region was amplified by PCR. The backward primer MCH4-1 (SEQ ID NO: 35) was designed in such a manner that it should hybridize to the nucleotide sequence encoding 5′ end of human μ chain constant region exon 4 and comprise the nucleotide sequence of 3′ end of humanized PM1 antibody single-chain Fv (SEQ ID NO: 23). The forward primer MCH4-2.1 (SEQ ID NO: 36) was designed in such a manner that it should hybridize to the nucleotide sequence encoding the extracellular region of human membrane-type μ chain constant region and comprise the restriction enzyme HindIII recognition site. PCR was conducted under the similar conditions to those of the above-mentioned manner using the two kinds of above-mentioned primers, and cDNA obtained from human myeloma cell-line CL-4 cells by common methods as the template DNA. The PCR product was purified using 1.5% low melting point agarose gel.

Next, the gene encoding humanized PM1 antibody single-chain Fv was amplified in the same manner. PCR was conducted under the same condition as mentioned above using EF1 (SEQ ID NO: 28) as the backward primer, SCP-Mu (SEQ ID NO: 37) as the forward primer, and pTMT-scFv as template DNA. The forward primer SCP-Mu was designed in such a manner that it should hybridize to the nucleotide sequence encoding single-chain Fv C terminus shown in SEQ ID NO: A, and comprise a nucleotide sequence that was complementary to the 5′ end of the μ chain partial sequence gene amplified by PCR. The PCR product was purified in the same manner.

Using the method shown in Example 2-3), the full-length cDNA fragment encoding shPM1-MCH4 was amplified. Namely, 100 ng each of the above-mentioned DNA fragments were assembled by primary PCR, and then 100 pmol each of the backward primer EF-1 (SEQ ID NO: 28) and forward primer MCH4-2.2 (SEQ ID NO: 38) was added to

amplify the full-length cDNA fragment. The forward primer MCH4-2.2 was designed in such a manner that it should hybridize to the 3′ end of the nucleotide sequence encoding human membrane-type μ chain partial sequence amplified above, and comprise the nucleotide sequence encoding FLAG peptide, two stop codons, and restriction enzyme NotI recognition site.

After purifying the PCR product with 1.5% low melting point agarose gel, it was digested with restriction enzymes EcoRI and NotI, and was inserted into pSFLAG vector to obtain shPM1-MCH4 expression vector pTMT-shPM1F-MCH4 (Fig. 7). Moreover, using methods as described in Example 5, single-chain Fv having a bivalent variable region, and shPM1- MCH4-BvGS3 (SEQ ID NO: 39) expression vector pTMT-shPM1FM-BvGS3 were obtained.

Example 8: Screening of STX561 cDNA library by the TMT method using the shPM1-kappa expression vector (Fig. 7)

1. Preparation of STX561 cDNA library

mRNA from the mouse hematopoietic stromal cell line STX561 was prepared by the usual method and the cDNA synthesized from this was inserted into the TMT expression vector shPM1-kappa to prepare STX561 cDNA library. cDNA library was prepared using cDNA synthesis kit (STRATAGENE, cDNA synthesis kit). Basically, the protocol of the cDNA synthesis kit of STRATAGENE was followed, with the modifications mentioned below. Namely, Superscript II of GIBCO-BRL was used as the reverse transcriptase, NotI-dT primer (Pharmacia Biotech, primer attached to 1st strand cDNA synthesis kit) as the primer for the first synthesis, HindIII-SmaI site adapter as the adapter added to the 5′ terminus of cDNA, and Size sep 400 Spun Column of Pharmacia Biotech as the column for size fractionation.

Specifically, the cDNA library was prepared as follows: The starting material was 5 μg of mRNA, and first, a first strand was synthesized from 3′ poly A tail by reverse transcriptase (Superscript II, GICO-BRL) using NotI-dT primer (Pharmacia Biotech, primer attached to 1st strand cDNA synthesis kit). Next, after synthesizing the second strand with DNA polymerase, both

ends of the cDNA were blunt-ended, and the HindIII-SmaI site adaptor (Takara) was added. After digesting both ends with HindIII and NotI, size-fractionation (Pharmacia Biotech, Size sep 400 Spun Column) was done to remove cDNA fragments with 0.5 kb or less in size. The collected cDNA was inserted into the HindIII-NotI sites of TMT expression vector shPM1-kappa, and the vectotr was introduced into *E. coli* DH10B (electroMAX DH10B, GIBCO-BRL) by electroporation method to prepare the STX561 cDNA library.

STX561 cDNA library was pooled by separating into 1000 clones/pool, and two pools thereof (pool no.:#kappa-1, #kappa-6), 2000 clones in total were used for screening by the TMT method.

2. Screening of STX cDNA library by panning

1) Transfection into COS-7 cells

2 μg each of the plasmid DNA prepared from #kappa-1 and #kappa-6 were transfected into COS-7 cells using FuGENE™6 (Boehringer-Mannheim).

Namely, COS-7 cells seeded in 1 x 10^5 cells/well (6-well plate) on the day prior to the transfection were cultured overnight under the conditions of 37℃ and 5% CO_2 in 2 ml of 10% fetal calf serum-containing DMEM culture medium (GIBCO-BRL). On the day of transfection, 6 μl FuGENE™ was added to 0.1 ml of serum-free DMEM culture medium and incubated for 5 min at room temperature, then mixed with 2 μg plasmid DNA, and incubated for further 15 min at room temperature. Next, this mixture was added to the above COS-7 cells, and incubated for 3 days under the conditions of 37℃ and 5% CO_2.

2) Preparation of the panning dish

A panning dish coated with goat anti-mouse IgG antibody (Dainippon Seiyaku, goat anti-mouse IgG (H + L chains)) was prepared according to the method of "Seed, B. et al., Proc. Natl. Acad. Sci. USA. (1987) 84, 3365-3369". Namely, goat anti-mouse IgG antibody was added to 50 mM Tris-HCl (pH9.5) to 10 μg/ml. The antibody solution thus prepared (3 ml) was added to 60 mm-diameter

cell-culture dish, and incubated at room temperature for 3 hours. After washing three times with 0.15M NaCl solution, PBS containing 5% fetal calf serum, 1 mM EDTA, and 0.02% NaN_3 was added, and then, after blocking, the panning was done as follows.

3) Panning

COS-7 cells transfected as mentioned above, were detached from the plate with PBS containing 1 mM EDTA, washed once with PBS containing 5% fetal calf serum, suspended in 50 µl of FACS buffer (PBS containing 2% fetal calf serum and 0.05% NaN_2).

Soluble-type IL-6R (2 µg) was added to the cell-suspension and incubated for 90 min on ice. Next, after washed twice with FACS buffer, the cells were suspended in 50 µl of FACS buffer. Then, 1.5 µg of mouse anti-IL-6 receptor antibody MT-18 was added to the cell suspension, and the suspension was incubated for 30 min on ice. Cells were washed twice with FACS buffer, suspended in 2 ml of PBS containing 5% fetal calf serum and 0.02% NaN_3, and added to a panning plate coated with goat anti-mouse IgG antibody.

After incubating the above-mentioned various COS-7 cells on panning plates at room temperature for about 2 hours, the plates were gently washed three times with PBS containing 5% fetal calf serum and 0.02% NaN_3, and plasmid DNA were collected from cells bound to the panning dishes using Hirts' solution (solution containing 0.6% SDS and 10 mM EDTA). Half of the collected plasmid DNA were transfected into 40 µl of *E. coli* DH10B (electroMAX DH10B, GIBCO-BRL) by the electroporation method, and after incubating for 1 hr in 1 ml of SOC culture-medium, 50 µl was sampled for the titer-check and seeded onto an LB-ampicillin (100 µg/ml) plate. On the other hand, the remaining culture was transferred to 500 ml of LB-ampicillin (100 µg/ml) liquid culture medium and cultured. After an overnight culture, plasmid DNA were prepared by plasmid DNA purification kit (Plasmid-Maxi, QIAGEN) and cryopreserved at -20℃.

A 1 µg portion of each of the plasmid DNA obtained per pool was re-transfected into COS-7 cells using 3 µl of FuGENE™6 (Boehringer-Mannheim), and the second panning, and recovery and

preparation of the plasmid DNA were done as mentioned above.

3. Analysis of the nucleotide sequence and deduced amino acid sequence of the obtained cDNA clone

Following the first and second panning, colonies were randomly collected from the plates for titer checking, and after culturing each in 2 ml of LB-ampicillin (100 μg/ml) liquid culture medium, plasmid DNAs were prepared. Next, one more cDNA insert was screened by restriction enzyme analysis using SmaI and NotI, sequencing was done from the 5'side, and as a result of analyzing their nucleotide sequence and deduced amino acid sequence, it was revealed that genes of membrane-bound proteins could be selectively screened by using the TMT method. The results are shown in Table 1.

Table 1

First Panning

Pool name (1000 clones/pool)	Analyzed clone number	Clone number having transmembrane regions	Details (insert size, amino acid residue number, transmembrane region number)
kappa-1	11	1	Cytochrome oxidase (0.75 kb, 44aa, 1TM)
kappa-6	7	3	NADH-dehydrogenase (1.7 kb, 88aa, 2TM) NADH-dehydrogenase (1.7 kb, 88aa, 2TM) ATP-synthase (0.85 kb, 42aa, 1TM)

Second Panning

Pool name (1000 clones/pool)	Analyzed clone number	Clone number having transmembrane regions	Details (insert size, amino acid residue number, transmembrane region number)
kappa-1	11	4	ATP-synthase (0.85 kb, 42aa, 1TM) ATP-synthase (0.85 kb, 42aa, 1TM) ATP-synthase (0.85 kb, 42aa, 1TM) Cytochrome oxidase (0.75 kb, 44aa, 1TM)
kappa-6	11	3	NADH-dehydrogenase (1.2 kb, 81aa, 2TM) NADH-dehydrogenase (3.5 kb, 58aa, 2TM) Poly T (0.9 kb, 35aa, 1TM)

From the first panning, known membrane-bound proteins, cytochrome oxidase (1 clone), NADH-dehydrogenase (2 clones), and ATP-synthase (1 clone) were obtained. On the other hand, from the second panning, known membrane-bound proteins, cytochrome oxidase (1 clone), NADH-dehydrogenase (2 clones), and ATP-synthase (3 clones) were obtained. All mentioned above are membrane-bound proteins localized in the mitochondria inner-membrane. For example, ATP-synthase is known to be one-transmembrane-type, cytochrome oxidase to be two-transmembrane-type, and NADH-dehydrogenase to be fifteen-transmembrane-type proteins. These results reveal that TMT method enables not only the isolation of type I membrane-bound proteins, but also proteins having multiple transmembrane regions.

A clone of poly T sequence obtained in the second panning is probably due to insertion of the cDNA comprising poly A in the opposite direction. Poly T is translated into an amino acid sequence in which phenylalanines, which are hydrophobic, are tandemly aligned, and is believed to have been isolated since it is extremely rich in hydrophobicity.

In addition, the percentage of membrane-bound proteins contained in the collected clones is higher in the second panning

than in the first panning. This shows that membrane-bound proteins are selectively condensed by repeating pannings.

Thus, in an actual cDNA library screening system, the TMT method was revealed to be an effective method for selectively cloning type I membrane-bound proteins and membrane-bound proteins comprising multiple transmembrane regions.

Industrial Applicability

Due to structural problems, it is believed that antibody molecules cannot easily exert their antigen-binding activity when they are in states where they are accumulated on the cell membrane in the secreting process, and where they are agglutinated due to unnatural structures with fusion proteins and due to the amino acid composition. Therefore, as in the present invention, cells functionally expressing antibody fusion proteins on the cell-surface can be selectively screened by using a panning plate prepared using an antigen recognizing an antibody. Namely, the present invention provided a method of extremely selectively cloning genes encoding cell membrane-bound proteins, by effective removal of cells having fusion proteins with little or no antigen-binding activity on the cell surface.